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Human interleukin 1β fused to the human growth hormone signal peptide is *N*-glycosylated and secreted by Chinese hamster ovary cells

(Recombinant DNA; eukaryotic expression vectors; synthetic gene; rerouting)

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SUMMARY

A hybrid gene consisting of the sequences coding for the signal peptide of human growth hormone and the mature form of interleukin-1β (IL-1β) was chemically synthesized. This sequence was inserted into a eukaryotic expression vector and introduced into Chinese hamster ovary cells. The resulting stably transformed cell lines produced large amounts of recombinant IL-1β, which was secreted into the culture medium mainly as a 22-kDa form. Expression in the presence of tunicamycin, an inhibitor of *N*-glycosylation, led to the complete disappearance of the 22-kDa form and the appearance of a new form of 17.5 kDa, indicating that the hybrid protein had been both processed and *N*-glycosylated. However, transformed cells producing mature IL-1β without a signal peptide produced the predicted 17.5-kDa nonglycosylated form. These results suggest that fusion to a heterologous leader sequence allowed IL-1β to be translocated across the membrane of the endoplasmic reticulum and to be transported and secreted by the exocytotic pathway.

INTRODUCTION

The IL-1 polypeptides are pleiotropic mediators which play a fundamental role in inflammatory and immune responses (Oppenheim et al., 1986). Two quite distinct IL-1 molecules, designated IL-1α and IL-1β, have been well characterized (March et al., 1985). Although IL-1 was originally detected in the supernatant of activated monocytes, it is now clear that a wide variety of cell types can produce it, as a response to various extracellular stimuli (Oppenheim et al., 1986). Both IL-1α and IL-1β are expressed initially as intracellular 31-kDa precursor polypeptides with IL-1β being the predominant species (March et al., 1985). Stimulated monocytes have been found to contain up to 5% of mRNA coding for IL-1β, with concomitant high levels of intracellular IL-1β protein (Webb et al., 1985). In addition, proteolytic processing of the precursor occurs, followed by secretion of a soluble mature form of active 17.5-kDa IL-1β corresponding to the 153-aa C-terminal end of the precursor (March et al., 1985). The precise cellular site of cleavage and the mode of secretion of the molecule remain unknown. Examination of the polypeptide reveals the absence of a classical N-terminal or internal hydrophobic signal peptide. Studies of activated

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; hGH, human growth hormone; IL-1, interleukin 1; MTX, methotrexate; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; re, recombinant; SDS, sodium dodecyl sulfate; Tu, tunicamycin; u, unit(s); UWGCG, University of Wisconsin Genetic Computing Group.

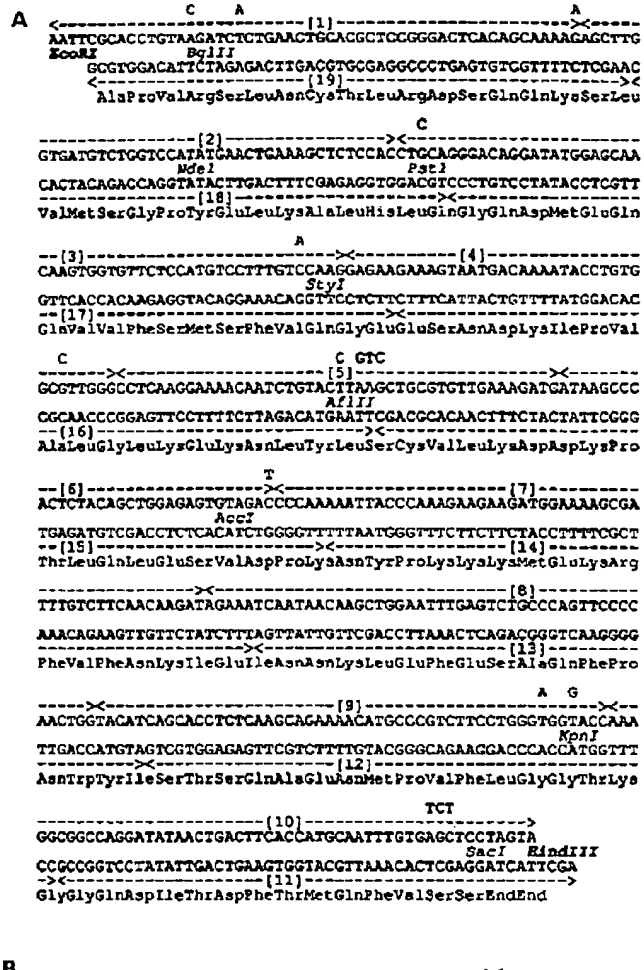

Fig. 1. Synthetic DNAs. Oligos were synthesized on a Biosearch 8700 Synthesizer using β-cyanoethyl-protected *N,N*-diisopropylphosphoramidites (Biosearch) and purified by 8 M urea/12% PAGE. (A) Sequence of the *IL-1β* gene. Numbers in brackets identify the 19 oligos used in gene assembly. Unique restriction sites are shown together with the sites reconstituted on ligating into the vector (bold-face letters). Original nt are shown above the coding strand. Apart from oligos 1 and 11, the oligos (1 μg) were phosphorylated either in complementary pairs or in a group of three (4 + 5 + 16) in a standard ligation mixture containing 50 mM Tris · HCl pH 7.8/10 mM $MgCl_2$/5 mM dithiothreitol/1 mM ATP/10 u of T4 polynucleotide kinase (1 μl) in a total volume of 11 μl. After 30 min at 37°C, the mixtures were heated at 65°C for 10 min. The solutions were pooled, oligos 1 and 11 were added and the mixture was heated for 10 min at 65°C. It was allowed to cool to 22°C, T4 DNA ligase (1 μl, 5 u) was added and the mixture was incubated for 10 min at 22°C. The synthetic gene was purified by 5% nondenaturing PAGE and the desired 469-bp fragment was electroeluted overnight into 40 mM Tris · acetate pH 7.9/1 mM EDTA/0.1% SDS. The DNA was further purified by DE-52 column chromatography with elution by 1.5 M NaCl/50 mM Tris · HCl pH 7.6/5 mM EDTA followed by ethanol precipitation. It was ligated into M13mp18 previously digested by *Eco*RI + *Hin*dIII and cloned into *E. coli* JM103. Clones were analysed by restriction enzyme digestion, followed by dideoxy sequencing in both directions. (B) Sequence encoding the hGH signal peptide. The 102-bp sequence coding for the hGH signal peptide flanked by *Hin*dIII and *Eco*RI sites was constructed from four synthetic oligos enzymatically phosphorylated at the internal junctions, ligated and purified as described above. The hGH signal peptide-IL-1β sequence has been deposited with GenBank under number M35049.

human monocytes have demonstrated that IL-1β is absent from the secretory apparatus of the cell, i.e., ER, Golgi apparatus and secretory vesicles (Bayne et al., 1986; Singer et al., 1988). Kostura et al. (1989) have shown that pre-IL-1β convertase activity is present in the cytosol of monocyte-like cells but not in a variety of blood lymphocytes and fibroblast cell lines. These data suggest that IL-1β retains its own monocyte-specific mechanism of processing and secretion, which differs from the typical secretory pathway in mammalian cells. When transfected with cloned full-length cDNAs of IL-1β mammalian cells have proved to be relatively inefficient in secreting mature IL-1β into culture media. This can be explained by the absence in these cells of the convertase activity identified in monocytes (Kostura et al., 1989). We decided to test whether a fusion between IL-1β and a heterologous signal peptide would allow the efficient secretion of mature IL-1β from transfected cells. We chose to express a synthetic gene encoding only the 153-aa C-terminal part of the protein as previously used by Joseph-Liauzun et al. (1990) in *Escherichia coli*. It was fused either to a synthetic sequence encoding the hGH signal peptide or directly to an initiator ATG. The aim of this study was the synthesis and expression of the two chimeric genes and the establishment of transformed CHO cell lines producing large amounts of two forms of mature IL-1β.

RESULTS AND DISCUSSION

(a) Gene design and assembly

The sequence of the synthetic *IL-1β* gene (Fig. 1A) corresponds to the natural genomic sequence, with a small number of codon modifications to have several unique restriction sites available for screening clones and for mutagenesis experiments. A total of 16 changes in 469 nt were made. The gene was flanked by *Eco*RI and *Hin*dIII restriction sites. A strategically placed unique *Bgl*II site permitted easy insertion of either a eukaryotic signal peptide or an ATG start codon plus the appropriate control sequences. At the 3' end, a *Sac*I site adjacent to the *Hin*dIII site facilitated the introduction of a eukaryotic polyadenylation signal. The length of the single-stranded oligos chosen (35–58 nt) was a compromise between ease of assembly and minimization of possible mutations in the cloned DNA. Each oligomer was examined with the 'Fold' computer program in the UWGCG package (Zuker and Stiegler, 1981; Devereux et al., 1984) to avoid dyad symmetries. We found that the

most efficient assembly was obtained under the conditions described in Fig. 1. The assembled synthetic fragment was ligated into the polylinker region of M13mp18 and the phage was used to infect *E. coli* JM103. White plaques were screened by restriction analysis and one clone (mpIL-1β; Fig. 2) was selected for dideoxy sequencing in both directions and found to be correct. The same protocol was used to synthesize the sequence encoding the hGH signal peptide (Roskam and Rougeon, 1979; Fig. 1B), and to insert it into M13mp18 to give mphGHsp (Fig. 2).

(b) Expression vectors

The *Bgl*II-*Sac*I fragment from mpIL-1β was used in the constructions pSV1003 and pSV1008 as outlined in Fig. 2. The complete synthetic sequences of pSV1003 and pSV1008 were verified by subcloning the *Hin*dIII-*Bam*HI fragments in M13mp18 and sequencing by the dideoxy method. Plasmid pSV1003 directed the synthesis of the 153-aa mature form of human IL-1β fused to the 26-aa hGH signal peptide. In pSV1008, the synthetic sequence coding for mature IL-1β directly followed the ATG start codon.

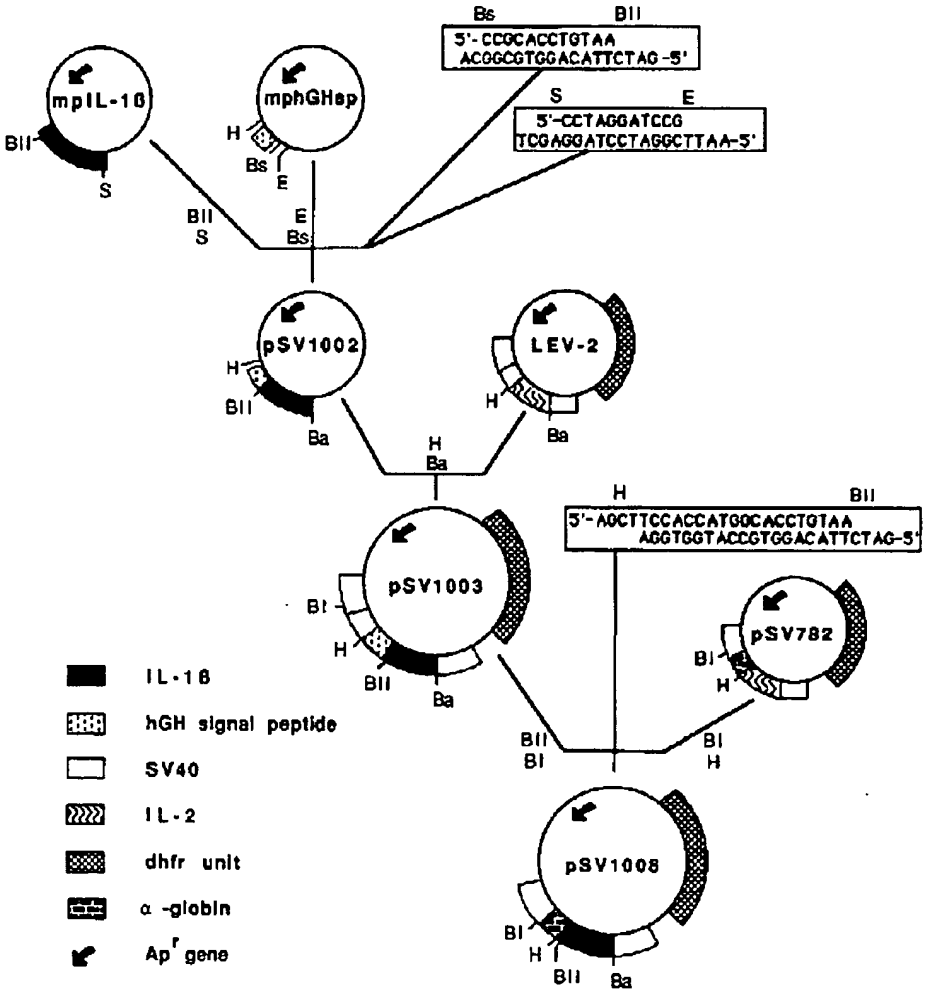

Fig. 2. Construction of expression vectors pSV1003 and pSV1008. The sequence coding for mature IL-1β was obtained from a *Bgl*II-*Sac*I fragment of mpIL-1β which was completed at the 5' and 3' ends by two synthetic oligos (boxed). It was ligated to a *Eco*RI-*Bsm*I fragment of mphGHsp containing the synthetic sequence coding for the hGH signal peptide to give pSV1002. The sequence coding for mature IL-1β preceded by the hGH signal peptide was inserted between the *Bam*HI and *Hin*dIII sites of eukaryotic expression vector LEV-2 to give pSV1003. Briefly, the vector LEV-2 contains the following elements: (*i*) an expression unit for the selectable marker (**dhfr unit**), derived from pSV2-*dhfr* (Subramani et al., 1981); (*ii*) a transcription unit for interleukin-2 (**IL-2**) flanked on 5' by *Hin*dIII and on 3' by *Bam*HI. This expression unit, derived from plasmids pclDV1 and pL1 of Okayama and Berg (1983), contains the early promoter, the late region introns and the late polyadenylation site of SV40; (*iii*) the pBR322 origin of replication and Ap^R (Ap^r) gene of pUC9 (Vieira and Messing, 1982). A third synthetic oligo designed to replace the hGH signal peptide by an ATG start codon (boxed) was introduced in pSV1003 together with the *Bgl*I-*Hin*dIII fragment of pSV782, a modified version of plasmid LEV-2. The resulting plasmid pSV1008 contains the second intron of mouse α-globin in front of the *IL-1*β sequence. Both plasmids pSV1003 and pSV1008 were optimized for translation initiation by using consensus sequences in front of the ATG codon as described by Kozak (1984). Ba, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; Bs, *Bsm*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I.

(c) Transient expression of IL-1β in CHO cells

The vectors pSV1003 and pSV1008 were tested in a transient-expression assay in CHO DHFR⁻ cells. As shown in Table I, both vectors allowed an efficient synthesis of IL-1β, but with a different distribution between the intracellular and extracellular compartments. With pSV1003, virtually all of the IL-1β was secreted into the culture medium. This shows that the hGH signal peptide placed in front of mature IL-1β results in nearly complete secretion of the protein. With pSV1008, 52% of the product was found in the culture medium. The mechanism by which this molecule is released from the cells is unknown. Transient expression of plasmid pSV2-cat (Gorman et al., 1982) in CHO DHFR⁻ cells, either alone or after cotransfection with pSV1008, resulted in a distribution between intracellular and extracellular CAT comparable to that observed with IL-1β (Table I). Recently, it has been suggested that CAT can be secreted by mammalian cells via an unusual pathway (Bunker and Moore, 1988). Since similar percentages of CAT and IL-1β were released into the medium, both molecules might use the same secretory pathway. We cannot exclude, however, that at least part of the IL-1β (and of the CAT) found in the culture medium is the result of cell lysis.

(d) Isolation of transformed CHO cell lines producing IL-1β

CHO DHFR⁻ cells were transformed with pSV1003 and pSV1008 to establish stable cell lines producing IL-1β. Fifty DHFR⁺ colonies were isolated from each transformation and assayed by ELISA for the presence of IL-1β in the supernatant. The clones which produced the highest amounts of IL-1β were subjected to a gene amplification procedure in increasing concentrations of MTX (Ferrara et al., 1987). Selection in 100 nM MTX yielded several highly productive cell lines. The best producers were clones 1003,26 and 1008,12, for which amplification resulted in a sixfold increase in IL-1β production. At 4×10^5 cells per 60-mm dish and four days of culture, these clones produced, respectively, 38 ng/ml and 44 ng/ml of IL-1β before amplification, vs. 236 ng/ml and 265 ng/ml after amplification. The levels of production were much higher than those described previously for mammalian systems, such as natural secretion by activated monocytes (Kronheim et al., 1985), hamster fibroblasts transformed with cloned full-length cDNAs (Young et al., 1988) or mouse L cells transformed with total human DNA (Corbo et al., 1987).

(e) Analysis of recombinant IL-1β

To analyse the IL-1β produced by the cell lines 1003,26 and 1008,12, [^{35}S]methionine-labeled proteins were immunoprecipitated using an anti-human IL-1β antiserum (Cistron) (Fig. 3). A predominant 22-kDa form and two minor 21-kDa and 17.5-kDa forms were immunoprecipitated from cell line 1003,26 supernatants (Fig. 3, lane a). Since a potential *N*-glycosylation site is present near the N terminus of mature IL-1β, the difference between the IL-1β produced in CHO cells and that from *E. coli* (Fig. 3, lane f) was probably due to the addition of *N*-linked oligosaccharides to the protein in CHO cells. The absence of cleavage of the signal peptide fused to IL-1β would have resulted in a hybrid 20.5-kDa protein. An inhibitor of *N*-glycosylation, Tu (Sigma; Takatsuki et al., 1975), was added to the culture medium during labeling, and immunoprecipi-

TABLE I

Transient expression of pSV1003, pSV1008 and pSV2-cat in CHO DHFR⁻ cells

Plasmid[a]	IL-1 (ng/dish)[b]		IL-1[c] (% secreted)	CAT (ng/dish)[b]		CAT[c] (% secreted)
	Intracellular	Medium		Intracellular	Medium	
pSV1003	2	62	97	—	—	—
pSV1008	73	79	52	—	—	—
pSV2-cat	—	—	—	1.87	1.12	39
pSV1008 + pSV2-cat	39	30	43.5	0.84	0.95	55

[a] Plasmid pSV2-*cat* is from Gorman et al. (1982); other plasmids are described in Fig. 2. Transient expression of plasmids was performed in a DHFR-deficient CHO cell line (CHO DHFR⁻) (Urlaub and Chasin, 1980) by use of the DEAE-dextran method as described (Lopker et al., 1983). Transformation of CHO DHFR⁻ cells, selection of transformed clones and gene amplification in increasing concentrations of MTX were performed as described earlier (Ferrara et al., 1987). Cell-culture products were from Gibco.

[b] Subconfluent cultures of CHO DHFR⁻ cells were transfected with 5 μg of DNA of single plasmids per 60-mm dish or cotransfected with a mixture of 5 μg of pSV1008 and 5 μg of pSV2-*cat*, in the presence of DEAE-dextran. Culture media were collected 65 h after transfection and clarified by centrifugation. Cell monolayers remaining on the dishes were washed twice with PBS (Dulbecco and Vogt, 1954), scraped off with a rubber policeman in 3 ml of PBS and sonicated. Cell extracts were spun at 10000 rpm for 15 min at 4°C and the pellets were discarded. All resulting supernatants and lysates were used immediately or stored at 4°C until testing for IL-1β (ELISA, Cistron, Pine Brook, NJ) or for CAT (ELISA, 5 Prime–3 Prime, West Chester, PA).

[c] $\%\ \text{secreted} = \frac{\text{medium}}{\text{intracellular} + \text{medium}} \times 100.$

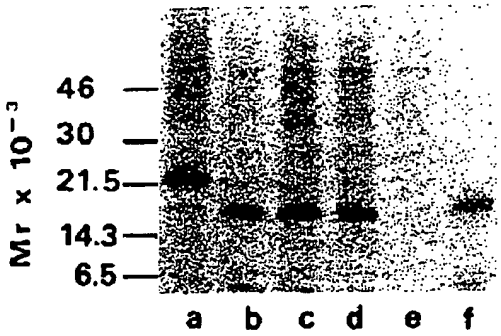

Fig. 3. Analysis by immunoprecipitation of IL-1β secreted by 1003,26 and 1008,12 cell lines. The 1003,26 and CHO DHFR cells were labeled with [^{35}S]methionine for 16 h and the 1008,12 cells for 48 h as follows: cells were seeded in 60-mm dishes at a density of 10^6 cells/dish. 24 h later, cells were washed twice with PBS and preincubated in methionine-free medium containing 0.2% of dialyzed fetal-calf serum, 300 μg/ml *L*-glutamine and 150 μg/ml *L*-proline. After 2 h, the medium was removed and replaced by the same medium (2 ml) containing [^{35}S]methionine (200 μCi, 37 TBq/mmol, Amersham) with or without Tu (10 μg/ml). Culture media were collected after 16 h or 48 h and clarified by centrifugation at 1000 rpm (10 min, 22°C). Labeled supernatants (200 μl) were incubated for 2 h at 4°C with control rabbit antiserum (10 μl) in the presence of fixed *Staphylococcus aureus* cells (Pansorbin-Calbiochem, 200 μl). The mixture was centrifuged for 5 min at 5000 rpm at 4°C, and rabbit polyclonal antiserum raised to human IL-1β (Cistron, 5 μl) and Pansorbin (200 μl) were added to the supernatant. Incubation was continued for 16 h at 4°C with agitation. The immunoprecipitates were further processed as described by Kessler et al. (1981) and modified by Vita et al. (1990). Aliquots were applied to 0.1% SDS/20% polyacrylamide Phastgels via the Phast system (Pharmacia LKB Biotechnology). Gels were dried and exposed to Hyperfilm-β max (Amersham). Lanes: a–e, culture supernatants of 1003,26 cells (a and b), 1008,12 cells (c and d), or CHO DHFR$^-$ cells (e), labeled in the absence (a, c and e) or in the presence (b and d) of Tu. Lane f, *E. coli*-derived [^{125}I]reIL-1β (4.44–9.25 MBq/μg, New England Nuclear).

tation was performed as described above. In this case, the disappearance of the two high-M_r bands leaving a unique 17.5-kDa band (Fig. 3, lane b) strongly indicates that IL-1β was indeed secreted from 1003,26 cells in an *N*-glycosylated form. In contrast, when 1008,12 cells were used, a major 17.5-kDa band was detected in the presence or absence of Tu (Fig. 3, lanes c and d), indicating that, in this case, the predicted nonglycosylated form of IL-1β was produced. Glycosylation of a secreted, nearly complete form of mature IL-1β in *Saccharomyces cerevisiae* has also been reported (Baldari et al., 1987); in this case, the protein was synthesized from a DNA coding for the mature form of IL-1β lacking the first four aa and fused to the presumptive signal peptide sequence of the *Kluyveromyces lactis* toxin gene. Abnormal glycosylation of a protein has also been reported by Eskridge and Shields (1986) who constructed a fusion protein between the N-terminal part of preproinsulin and bacterial CAT. Following in vitro translation, CAT underwent *N*-glycosylation, presumably at a single site. The results presented here suggest that fusion of mature IL-1β to a heterologous signal peptide allowed the protein to cross the membrane of the rough endoplasmic reticulum and to follow the pathway of a typical secretory protein. Transport of IL-1β to the ER and Golgi apparatus after signal cleavage allowed full glycosylation, presumably at a single potential site at Asn^{123}, (Asn^7, Fig. 1A) and, finally, secretion.

(f) Biological activity of IL-1β

The biological activities of both forms of recombinant IL-1β were tested by measuring the proliferation of the IL-1-dependent lymphocyte cell line D10.G4.1 in the presence of concanavalin A (Kaye and Janeway, 1984). IL-1β synthesized by CHO cells transfected with plasmids pSV1003 or pSV1008 was found to be biologically active (Table II). The results confirmed the different rates of secretion for the two different IL-1β molecules.

(g) Conclusions

We describe the isolation of CHO cell lines which efficiently produce human IL-1β. A fusion between the hGH signal peptide and the mature C-terminal part of IL-1β resulted in virtually complete secretion of a glycosylated form of IL-1β, which was biologically active. These results show that part of the IL-1β molecule can be efficiently rerouted to the classical secretory pathway by the addition of a signal peptide. When the biologically active part of IL-1β was preceded only by a methionine and synthesized in CHO cells, a considerable percentage of the IL-1β produced was quite unexpectedly found in the culture medium. A similar result has been obtained for the bacterial enzyme CAT produced in mammalian cells (Bunker and Moore, 1988). This suggests that certain molecules seem to be exported from the cell – either actively or passively – directly from the cytoplasm. Since the IL-1β precursor molecule is not secreted from mammalian cells, cleavage of this precursor could be the first step in the secretion pathway of this cytokine.

TABLE II

Biological activity of recombinant IL-1β

Plasmid[a]	IL-1 (x 10^3 u/dish)[b]		IL-1[c] (% secreted)
	Intracellular	Medium	
pSV1003	2.1	60.2	97
pSV1008	12.5	14.6	54

[a] See footnote a of Table I.

[b] Transient expression in CHO DHFR$^-$ cells was performed as described in Table I, except that 12.5 μg of DNA per 100-mm dish were used. The biological activity of IL-1β was assayed in the D10.G4.1 proliferation assay (Kaye and Janeway, 1984). The IL-1β used as a control (10^8 u/mg) was obtained from the National Institute for Biological Standards and Control (London, U.K.).

[c] See footnote c of Table I.

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